Evaluation of the influence of Different Culture Media and Atmospheric Conditions on *ex vivo* Biofilms of Individuals with Advanced Periodontitis

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Abstract

Aims: To assess the effects of different nutritional and atmospheric conditions on the density and bacterial diversity of *ex vivo* biofilms of individuals with advanced periodontitis.

Materials and methods: Subgingival biofilm samples were collected from volunteers with advanced periodontitis and immediately sent for cultivation of *ex vivo* biofilms. The biofilms were cultured in Brain Heart Infusion (BHI) + 1% hemin + 5% sheep blood or in BHI + 5% bovine serum; under anaerobic or capnophilic conditions, for time intervals of up to 7 and 14 days. The microbial composition was analyzed by Checkerboard DNA-DNA hybridization.

Results: Atmospheric condition had a greater influence on bacterial counts than the type of culture media used. Higher bacterial counts were observed in anaerobic than in capnophilic atmosphere condition, irrespective of the media used and the time of growth. The type of medium influenced diversity. Although, the 40 species assessed have been identified in all tested conditions, in capnophilic atmosphere and serum-enriched-media only 12 bacterial species were identified, and the counts of most of them were very low.

Conclusions: The two different nutritional and atmospheric conditions tested allowed the growth of multispecies *ex vivo* biofilms. Anaerobic growth in enriched blood media provided the highest bacterial counts and the richest biofilm diversity.

Keywords: Periodontitis, biofilm, atmospheric conditions, periodontal diagnosis

Introduction

Current knowledge about the microbial etiology of periodontal diseases and the microbiological effects of different periodontal treatments is mainly based on the 40 microorganisms that can be analyzed by the checkerboard DNA-DNA hybridization technique (Socransky *et al*., 1994; Socransky *et al*., 2004). Certain microbial species analyzed by this method, including *Aggregatibacter actinomycetencomitans*, *Porphyromonas gingivalis, Tannerella forsythia* and species from the genus *Treponema*, are considered true periodontal pathogens, while the orange complex comprises several putative periodontal pathogens (Consensus report, 1996; Teles *et al*., 2006; Teles *et al*., 2013).

More recently, it has been recognized that a greater diversity of bacterial species can colonize the subgingival environment (Abusleme *et al*., 2013; Perez-Chaparro *et al*., 2014; Oliveira *et al*., 2016). Many of these microorganisms have been suggested to be potential periodontal pathogens, including *Porphyromonas endodontalis*, *Filifactor alocis*, *Prevotella tannerae*, *Selemonas sputigena*, *Dialister pneumosintes*, *Atopobium parvulum*, *Prevotella oris* and *Prevotella denticola*, as well as other taxa recently cultivated, such as

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those from the genera *Fretibacterium* and *TM7* (Kumar *et al*., 2005; Dahlen and Leonhardt, 2006; Vartoukian *et al*., 2013, Perez-Chaparro *et al*., 2014; He *et al*., 2015; Bor *et al*., 2020). The definitive association of microorganisms with periodontal health or disease, and nomenclature of newly identified bacterial species depend on their cultivation. Thus, detailed studies using bacterial culture, including *in vitro* and *ex vivo* biofilm models are important and have regained strength in recent years. For instance, through technological advances in culture methods, bacterial species of the environment that had previously not been cultivated, have now been successfully grown using the culturomic method (Lagier *et al*., 2017). The growth of species that have not yet been cultivated may require different nutritional and atmospheric conditions. The composition of different media may play an important role in preventing or fostering the growth of specific bacterial species (Prakash *et al.* 2013). Also, many bacterial species found in the periodontal biofilm are strict or facultative anaerobic and would not survive in a capnophilic atmospheric condition.

Thus, the objective of this study was to evaluate the effect of different culture media, atmospheric conditions and time period of bacterial growth on the composition of *ex vivo* biofilms of individuals with advanced periodontitis.

Materials and methods

The protocol was approved by the Institutional Review Board of UNG (Clinical Research Ethics Committee, CAAE: 40339114.7.0000.5506), and was conducted with individuals who voluntarily sought dental care at Guarulhos University. Three volunteers with advanced periodontitis were selected. All eligible volunteers were informed about the nature, potential risks and benefits of their participation in the study. They were invited to sign a term of free and informed consent, and received a copy of it. All volunteers were in good general health and were diagnosed with generalized periodontitis stage 3 or 4, grade C, based on the current classification of the European Federation of Periodontology (Papapanou *et al*., 2018).

Inclusion and exclusion criteria

The inclusion criteria were: ≥ 20 teeth, ≥ 8 sites in different teeth with pocket depth (PD) ≥ 5mm, clinical attachment level (CAL) ≥ 3mm and bleeding on probing (BOP). Exclusion criteria were: previous subgingival periodontal treatment, use of systemic antibiotic in the last six months, pregnancy, breastfeeding, smoking, systemic diseases that could affect the progression of periodontal disease and the continuous use of anti-inflammatory medications.

Clinical examination

The clinical parameters evaluated were as follows: presence or absence (0/1) of dental biofilm, gingival bleeding, bleeding on probing (BOP) and suppuration; and measurements (mm) of pocket depth (PD) and clinical attachment level (CAL). Each parameter was evaluated at six sites per tooth (mesio-buccal, buccal, disto-buccal, disto-lingual, lingual and mesio-lingual) in all teeth of the arch, excluding third molars. PD and CAL were measured using a North Carolina-type periodontal probe (Hu-Friedy, Chicago, IL, USA) (Haffajee *et al*., 1983).

Microbiological evaluation

Two subgingival biofilm samples per subject were collected from one selected site with PD ≥5 mm, using individual mini-Gracey sterile curettes (Hu-Friedy). The first sample (from each subject) was placed in plastic tubes containing 100µl TE (10mM Tris-HCl, 1mM EDTA pH 7.6) and 100µl of 0.5M NaOH were added to each tube. These samples were then stored at -80°C until processing. The second sample was transferred to tubes containing 350µl of pre-reduced medium and immediately sent to the laboratory for the culture of *ex vivo* biofilms for a period of 14 days, as explained below. The microbiological composition of the biofilms was evaluated by checkerboard DNA-DNA hybridization for the presence, levels and proportion of 40 bacterial species.

Development of ex vivo biofilms

The biofilms were developed using Calgary Biofilm Devices (Ceri *et al*., 1999). Two 96-well plates were used, as illustrated in Figure 1, and were prepared using 20 µl of culture medium per well.

The culture media tested were (i) Brain Heart Infusion + 1% hemin + 5% defibrinated sheep blood; (ii) Brain Heart Infusion + 5% bovine serum. Half of the wells in each plate received culture medium (i) and the other half received culture medium (ii). Then, the inoculums containing the biofilm sample collected from each patient were gently vortexed before each aliquot be dispersed in the plates (5 µl per well). Each plate received a cover slip containing pegs, on which the biofilms were grown. Then, one plate was incubated under anaerobic, and the other under capnophilic conditions, both at 37°C. After four days of incubation, the cover plates containing the biofilms were transferred to a new 96-well plate containing fresh culture media; this was done on a daily basis during entire length of both time intervals. On the seventh and 14th days of biofilm growth, four pegs of each condition containing the *ex vivo* biofilms were removed from the cover slip and transferred to tubes containing 150µl TE (10mM Tris-HCl, 1mM EDTA pH 7.6). In addition, 100 µl of 0.5 M NaOH were added to each tube, and then they were stored in a freezer at -80 °C until the samples could be processed.

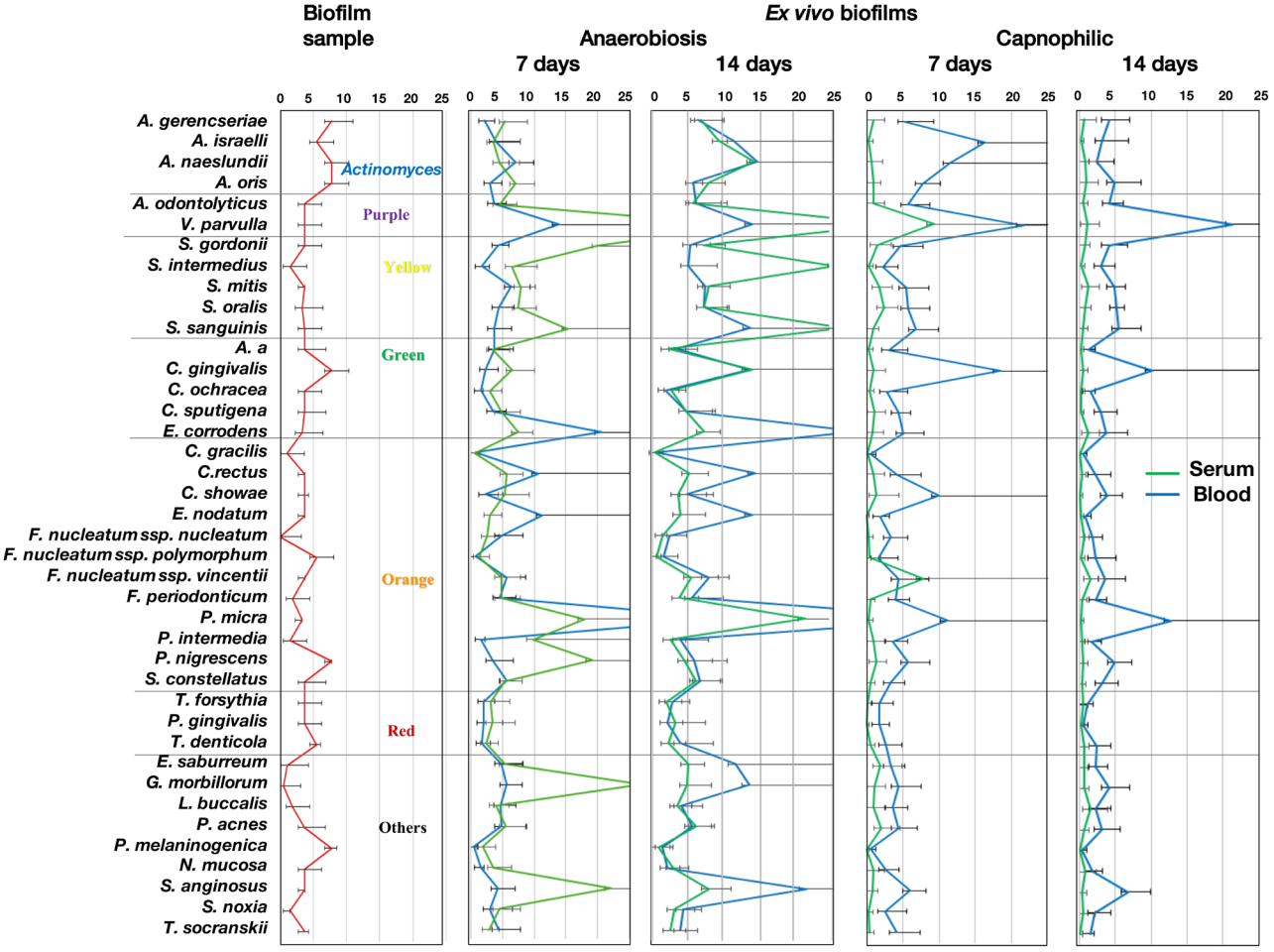

Figure 1. Mean counts (x105 cells) of the 40 bacterial species assessed in the initial biofilm samples, and in ex vivo biofilms cultivated in anaerobic and capnophilic conditions, fed with BHI enriched with fetal bovine serum or sheep blood, for time intervals of seven and 14 days, and the error bars indicate the standard errors of the mean. The species are ordered according to the microbial complexes.

Analysis of the samples by checkerboard DNA-DNA hybridization

The microbiological analysis was performed at the Laboratory of Microbiology of Guarulhos University (Socransky *et al.* 1994). Briefly, the biofilm samples were removed from the freezer -80°C, boiled in a water bath for 10 minutes and neutralized by adding 0.8 ml 5 M ammonium acetate. Each suspension was then deposited on one of the channels of a Minislot 30 apparatus (Immunetics, Cambridge, MA, USA) and transferred to the positively charged nylon membrane (Amersham Biosciences UK Limited, Buckinghamshire, England). The last two channels of the Minislot were occupied by control standards containing a mix of the DNA of all bacterial strains being investigated (Table 1) by the 40 probes, at concentrations corresponding to 10^5 and 10^6 cells of each bacterial species. The membrane was removed from Minislot 30 and the DNA therein was fixed by heating in an oven at 120 °C for 20 min. Subsequently, the membrane was pre-hybridized at 42° C for 1 hour in a solution containing 50% formamide (Vetec Química Fina Ltda, Rio de Janeiro, RJ, Brazil), 1% casein (Vetec), 15 M sodium citrate (JTBaker, Mexico, Mexico), 25 mM sodium phosphate (Na 2 HPO 4, Labsynth), and 0.5 mg / mL yeast RNA (Sigma Aldrich Química Brasil Ltda, São Paulo, SP, Brazil). The membrane was then positioned in Miniblotter 45 (Immunetics, Cambridge, MA, USA). Whole genomic DNA probe for 40 bacterial species, labeled with digoxigenin, were hybridized in individual lanes. Hybridization at 42°C occurred within a minimum of 20 hours. After hybridization, the membranes were washed at high stringent solution and the DNA probes were detected by using the antibody to digoxigenin conjugated with alkaline phosphatase and chemiluminescence detection. Signals were evaluated visually, by a calibrated examiner (kappa= 93%), by comparison with the standards lanes on the same membrane. Signals were converted to absolute counts recorded as: 0, not detected; 1, < 10^5 cells; 2, ~ 10^5 cells; 3, 10^5–10^6 cells; 4, ~ 10^6 cells; and 5, > 10^6 cells. The sensitivity of the assay was adjusted to permit the detection of 10^4 cells of a given species by adjusting the concentration of each DNA probe. The mean counts (10^5 cells) of individual bacterial species were initially

Table 1. Bacterial strains

Actinomyces gerencseriae ATCC 23840	*Campylobacter rectus* ATCC *33238(371)*
Actinomyces israelii ATCC 12102	*Campylobacter showae* ATCC 51146
Actinomyces naeslundii ATCC 12104	*Eubacterium nodatum* ATCC 33099
Actinomyces oris ATCC 43146	*Eubacterium saburreum* ATCC 33271
Actinomyces odontolyticus ATCC 17929	*Fusobacterium nucleatum ss nucleatum* ATCC 25586
Veillonella parvula ATCC 10790	*Fusobacterium nucleatum ss polymorphum* ATCC 10953
Streptococcus gordonii ATCC 10558	*Fusobacterium nucleatum ss vincentii* ATCC 49256
Streptococcus intermedius ATCC 27335	*Fusobacterium periodonticum* ATCC 33693
Streptococcus mitis ATCC 49456	*Parvimonas micra* ATCC 33270
Streptococcus oralis ATCC 35037	*Prevotella intermedia* ATCC 25611
Streptococcus sanguinis ATCC 10556	*Prevotella nigrescens* ATCC 33563
Streptococcus anginosus ATCC 33397	*Prevotella melaninogenica* ATCC 25845
Aggregatibacter actinomycetemcomitans ATCC 29523	*Streptococcus constellatus* ATCC 27823 (M32b)
Treponema socranskii D40DR2 (S1)[a]	*Tannerella forsythia* ATCC 43037(338)
Capnocytophaga gingivalis ATCC 33624(27)	*Porphyromonas gingivalis* ATCC 33277
Capnocytophaga ochracea ATCC 33596(25)	*Gemella morbillorum* ATCC 27824
Capnocytophaga sputigena ATCC 33612(4)	*Leptotrichia buccalis* ATCC 14201
Eikenella corrodens ATCC 23834	*Neisseria mucosa* ATCC 19696
Treponema denticola B1[a]	*Propionibacterium acnes* ATCC 11827
Campylobacter gracilis ATCC 33236 (1084)	*Selenomonas noxia* ATCC 43541

[a] From The Forsyth Institute, ATCC: American Type Culture Collection

averaged within each sample / *ex vivo* biofilm and then across samples / *ex vivo* biofilm. Then, mean counts was determined in each condition, and averaged across conditions at each time point.

Statistical analysis

Microbiological data were expressed as counts (levels) of 40 bacterial species evaluated. Initially, the mean levels of each species per sample/*ex vivo* biofilm was calculated, followed by the mean bacterial counts in each condition and then across conditions at each time point. Significant differences between the experimental groups and baseline were evaluated using Mann-Whitney and Kruskal-Wallis test. Statistical significance was set at 5%.

Results

The first biofilm sample collected from each volunteer was used as a control for the *ex vivo* biofilms. It was observed that *ex vivo* biofilms growth from each patient samples under anaerobiosis condition in blood enriched media reached microbial profiles similar to the original biofilm samples collected. For example: samples from patients one and two had high counts of all bacterial species; while samples from patient three had lower levels of bacteria. Similar results were observed for the *ex vivo* biofilms of each subject.

Figure 1 shows the mean counts ($x10^5$) of the species evaluated in three initial biofilm samples, and in all *ex vivo* biofilms cultivated in anaerobic and capnophilic conditions, fed with fetal bovine serum and sheep blood, for time intervals of seven and 14 days. When atmospheric conditions were compared, both allowed the development of all bacterial species evaluated. The red complex species, *Porphyromonas gingivalis*, *Treponema denticola and Tannerella forsythia* grew in both atmospheric conditions, but under capnophilic conditions both *P. gingivalis and T. forsythia* achieved very low mean of counts, mainly with serum nutrition (0.3 and 0.1 $x10^5$ and 0.5 and 0.3 $x10^5$ at the 7^{th} and 14^{th} days respectively). In the anaerobic condition, there were no statistical difference in the counts of *P. gingivalis* grown with serum or blood up to 14 days ($p>0.05$), however when compared with initial mean counts, *ex vivo* biofilms with seven days of growth, fed with blood, had statistically lower *P. gingivalis* counts ($p < 0.05$). The capnophilic condition benefitted the culture of *C. gingivalis* and *C. showae* in seven days, but all other species had higher counts in the anaerobic atmosphere ($p<0.05$). Under anaerobic conditions, at seven days of growth, the plates containing fetal bovine serum had higher microbial counts than those containing sheep blood ($p<0.05$). Whereas at 14^{th} days eight bacterial species achieved higher mean counts when they were incubated with medium enriched with sheep blood

than those incubated with serum ($p<0.05$). Although the capnophilic condition, in general, was worse than the anaerobic type relative to favoring the growth of all species, the biofilms cultivated with blood - even in the capnophilic condition - had better diversity and higher mean counts either in both seven and 14 days of culture, than those cultivated with serum. ($p<0.05$).

Table 2 shows the microbiological diversity achieved in each condition by the mean number of bacterial species detected. At baseline, the samples that were collected from periodontal sites were detected to contain an average of 38.5 bacterial species from the 40 probes used for checkerboard DNA-DNA hybridization. In the anaerobic condition, irrespective of nutrition or time interval of incubation, the same mean number of bacterial species were observed. However, under capnophilic conditions, the biofilms were significantly affected by nutrition. For instance, the least diversity was observed with serum nutrition under the capnophilic condition at 7 and 14 days with a mean number of 11 and 12.5 bacterial species, respectively.

Table 2. Mean number of bacterial species detected or not on biofilm samples collected at baseline and on cultivated *ex vivo* biofilms at different atmosphere and nutritional conditions.

	Baseline (n=3)		
	38.5(1,5)[a]		
Anaerobiose Blood 7 days	37(3)[a]	37(3)[a]	Capnophilic Blood 7 days
Anaerobiose Serum 7 days	37(3)[a]	11(29)[b]	Capnophilic Serum 7 days
Anaerobiose Blood 14 days	37(3)[a]	33.5(6.5)[a]	Capnophilic Blood 14 days
Anaerobiose Serum 14 days	37(3)[a]	12.5(27.5)[b]	Capnophilic Serum 14 days

Different letters mean the statistical differences ($p< 0,05$). For each condition a total of 12 *ex vivo* biofilms were analyzed.

Discussion

The results of this study suggested that different nutritional and atmospheric conditions had a different influence on the growth of *ex vivo* biofilms. The mean counts of all bacterial species were more affected by the atmospheric condition than by the different culture media used. However, biofilm diversity was affected by the nutritional factors. For instance, both blood and serum favored the growth of higher bacterial counts under anaerobic than capnophilic conditions, while media enriched with serum limited the bacteria diversity under capnophilic conditions. The fact that the bacterial levels were higher under anaerobic conditions was - to some extent - expected, since most of the species present in the subgingival pocket (*ex vivo* samples) are strict anaerobes. Nonetheless, bacterial species that are very sensitive to oxygen and difficult to cultivate, such as *Treponema ssp.* and *T. forsythia* were identified in the *ex vivo* biofilms, even under capnophilic conditions. These findings reinforce the notion that microorganisms growing in a biofilm structure can co-aggregate and give rise to a complex microbiota that is capable of changing the microenvironment and protecting each other from adverse conditions (e.g. oxygen levels) (Socransky and Haffajee, 2002; Socransky and Haffajee, 2005; Vartoukian *et al.*, 2013; He *et al.*, 2015). Previous investigations have also detected strict anaerobic pathogens of the red complex in oxygenated areas of the oral cavity, such as supragingival plaque, tongue and oral mucosa (Ximenez-Fyvie *et al.*, 2000; Mager *et al.*, 2003; Shibli *et al.*, 2008).

In terms of nutritional conditions, Periasamy *et al.* (2009) suggested that the addition of serum to agar plates could favor the growth of *Fusobacterium* species. This is an important observation for studies in oral microbiology, considering the important role of orange complex species in acting as a "bridge" between the early colonizers and the late colonizers from the red complex. Under the anaerobic condition and in the presence of serum, higher mean counts of seven bacterial species were detected than when sheep blood was used, after seven days of biofilms growth. However, after 14 days more species were found in higher counts in biofilms incubated with media enriched with blood rather than with serum, including *Fusobacterium ssp.* These data agree with the description of the ecological system of biofilm, where needed nutrients do not need to be supplied by the environment, because they may be the product of other species present in the biofilm (Socransky and Haffajee, 2002). For example, nutrition factors could be better supplied by the products of blood metabolism than directly by the serum. Comparing the nutritional and atmospheric conditions, only for *Fusobacterium sp vincentii*, in 7 days of growth under capnophilic atmosphere the media supplemented with serum was more effective in providing bacterial growth than blood. This showed that the effect of serum enriched medium would favor the cultivation of some *Fusobacterium ssp* only in a short period of time.

The Checkerboard DNA-DNA hybridization was chosen for the microbial analysis in this study because this technique has been validated in several previous studies (Lemos *et al.* 2020, Feres *et al.* 2015, Tamashiro *et al.* 2016, Soares *et al.* 2014). In addition, the importance

of the "microbial complexes" as biological markers for dysbiosis, symbiosis and rebiosis has been largely recognized. A recently published paper confirmed that even the studies using next generation sequencing techniques have confirmed the relevance of the 40 microorganisms of the microbial complexes in defining periodontal health or disease (Feres *et al*. 2021). Besides, the Checkerboard DNA-DNA hybridization technique allows the assessment of counts and proportion of a variety of microorganisms, which is very useful to assess the composition of the microbiota. For instance, clinical trials can analyze the change of proportions occurring after treatments, providing a good picture of the shifts occurring in the subgingival environment. For studies such as the present one, the technique allows to estimate the levels of the microorganisms growing in the biofilms, which is very helpful to estimate the microbial differences between the conditions.

An important aspect of this study design was that that only 3 biofilm samples were used to cultivate 96 *ex vivo* biofilms, under four different nutritional/atmospheric conditions, in two different time intervals. This allowed evaluation of the effect of each atmospheric/nutritional states on similar biofilm and supported the notion that different conditions may allow distinct profiles of the same biofilm sample. Another important piece of information that could be drawn from the results of the present study was that the model studied worked properly for *ex vivo* biofilm growth. Microbial species that are difficult to grow, such as species of the genus *Treponema* and some *Fusobacterium* species are often found at low levels in biofilm samples taken from patients, which make it difficult to conduct *in vitro* studies properly. This was the case of *F. nuc. sp. nucleatum*, which was present in very low levels in the patients' mouths, but it grew in the biofilms. One may think that it might have been identified species from DNA from dead bacterial cells; however, this was not possible because the biofilms were cultured from a small amount of cells, and from day four of growth they were transferred daily, to assure that only active cells were able to grow and be identified. Therefore, this experimental model, using an *ex vivo* biofilm, could be an important tool for future studies on bacterial interactions and to test preventive and therapeutic strategies for periodontal infections.

The limited number of conditions tested maybe considered a limitation of this study, but these results may help the conduction of studies using bacterial cultivation and guide other studies in this area.

In conclusion, the two different nutritional and atmospheric conditions tested provided the growth of multispecies *ex vivo* biofilms. Anaerobic growth in enriched blood media provided the highest bacterial counts and the richest biofilm diversity.

Acknowledgements

We acknowledge with thanks, the support provided by the Brazilian National Council for Scientific and Technological Development (CNPq), by granting the Young Researcher's scholarship to Caio J. Tanaka.

This research project did not receive any specific grant from funding agencies in the public, commercial, or nonprofit organizations.

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